Title: Characterization and Identification of Lactic Acid Bacteria in "Morcilla de Burgos"

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Abstract

A total of 176 lactic acid bacteria (LAB) isolated from a typical Spanish blood sausage called "morcilla de Burgos" were identified by means of phenotypic characteristics and 16S rDNA RFLP (ribotyping). LAB were isolated from "morcilla" of different producers and in different storage periods, that includes unpackaged, vacuum and modified atmosphere packaged "morcilla" and vacuum packed and pasteurised "morcilla". The knowledge of specific spoilage bacteria of "morcilla de Burgos" will be useful to design new preservation methods to extent the shelf-life of this product. Identification made according to phenotypic and biochemical characteristics shows the majority of the isolates were heterofermentative LAB (93.2%) and eight different bacterial groups could be distinguished (A-G). *W. viridescens* was the main species detected (42%). In addition, *Leuconostoc* spp. (23.9%), *W. confusa* (11.4%) and *Lactobacillus fructosus* (5.7%) species were found. Few strains were phenotypically missidentified as *Lb. sanfrancisco*, *Pediococcus* spp., *Lb. sakei/curvatus* and *Carnobacterium* spp. and 11 strains remained unknown. Most of the leuconostocs were identified as *Lc. mesenteroides* and *Lc. carnosum* species. Ribotyping shows a quite good correlation with phenotypic methods, although it has been possible to identify 15 different clusters. *W. viridescens* and leuconostocs were also the predominant LAB. Strains identified as *W. confusa* by phenotypic characteristics were resolved in *W. confusa* and *W. cibaria* by ribotyping. Neither *Carnobacterium piscicola* nor *Lb. sanfrancisco* were identified by means of genotypic method. All *Lb. fructosus* strains and some more included in different phenotypic groups (17 strains in total) could not be associated with any reference strain (cluster VII). Although some discrepancies exists the combination of phenotypic and genotypic methods led to a better identification and characterization of the strains isolated from "morcilla de Burgos".

53 **Keywords:** lactic acid bacteria, ribotyping, identification, blood sausages, spoilage

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Introduction

Blood sausages are very traditional meat products, which can be found all around Europe with slightly different composition. These kind of products although very popular, have not been studied in detail. In Spain “Morcilla de Burgos” is the most typical and popular blood sausage. It consists of a mixture of onion, rice, animal fat (mainly lard and sometimes tallow), blood, different spices and salt stuffed in a natural casing. The product is cooked for one hour at 94-95ºC, air cooled to 8-10ºC and finally chilled stored at 4ºC. Physicochemical and sensory characteristics of this product have been described in a previous work (Santos et al., 2003).

Lactic acid bacteria (LAB) contribute actively in the spoilage of “morcilla de Burgos”, where they have been identified as the main microbial group involved in the spoilage, especially in vacuum and modified atmosphere packaging (Santos et al, 2001), in the same way as many authors have reported for different meat products (Blickstard and Molin, 1983; Korkeala and Mäkelä, 1989; von Holy et al., 1991, 1992; Borch et al., 1996; Franz and von Holy, 1996; Korkeala and Björkroth, 1997). The typical sensory changes occurring in packaged “morcilla” are blowing of the packs, development of drip, slime formation and souring of the product. Nowadays no data is available about LAB species growing in this kind of blood sausage.

Although classical approach to bacterial identification based on morphological, physiological and biochemical features provides reasonable results and is easy to perform, in general these techniques are not always reliable for the identification of LAB (Stiles and Holzapfel, 1997). Genotypic methods have a higher discriminatory power and in this sense the efforts of the current bacterial taxonomy are

oriented to a polyphasic approach, which involves phenotypic and genotypic characterisation (Vandamme et al., 1996). Ribotyping technique that combines Southern hybridisation of chromosomal DNA fingerprints with the uses of Escherichia coli rRNA probes has revealed as a powerful tool in the classification of LAB (Rodtong and Tannock, 1993; Björkroth and Korkeala, 1996a, 1996b; Björkroth and Korkeala 1997; Björkroth et al., 1998; Lyhs et al., 2000; Björkroth et al., 2000, Satokari et al., 2000).

The aim of this work was to identify and characterise the LAB strains isolated from "morcilla" produced in Burgos region in order to identify the species responsible for the LAB spoilage of "morcilla". The isolates were initially classified according phenotypic and biochemical characteristics and further identified by ribotyping. Results from both identification methods were analysed and compared.

Material and Methods

Origin of the strains

One hundred and seventy-six strains of LAB were randomly selected out of 254 total LAB isolates from "morcilla" under different storage conditions. Sixty-six strains were isolated from blood sausages of eleven different producers just 24 h after elaboration process and before to be packaged, when the product suffers post-cooking contamination.

Ninety-two isolates came from preservation experiments of “morcilla” (Santos et al., 2001). Thirty seven strains were isolated from paper wrapped “morcilla” preserved under aerobic atmosphere, 31 came from vacuum “morcilla” and the remaining 24 strains were isolated from modified atmosphere packaged (MAP) “morcilla”. Unpackaged and packaged “morcilla” were kept at 4ºC during the storage time. In all cases, strains were isolated when LAB counts were over 6 log ufc/g and pH had decreased from 6.4 below to 5.0 and LAB were the dominant microflora in vacuum and modified atmosphere packaged product.

The last eighteen strains were isolated from vacuum-packaged “morcilla” which had been subjected to a mild pasteurisation. In this case, the product was pasteurised by packaging it under a low permeability film followed by a heat treatment in water at 75ºC for ten minutes. After pasteurisation, packages were cooled in an ice-water bath at 0ºC and stored at 4ºC for two months.

Microbial analysis

Twenty-five grams samples of “morcilla” were taken aseptically and homogenised with 225 ml of sterile Ringer's solution (Oxoid, Basingstoke, UK) for 2 min in a sterile plastic bag in a lab blender (Stomacher 400, Seward, London, UK). For LAB isolation, samples were plated on MRS agar (Oxoid) and the plates were incubated anaerobically at 6 % of CO_2, at 30°C for 2-3 days. Colonies were randomly selected from MRS plates containing less than 300 colonies and stroke to purify on MRS agar. All isolates were initially examined for Gram reaction and production of catalase and oxidase. Only Gram-positive, catalase-negative, oxidase-negative isolates were stored frozen at - 80°C in MRS broth (Oxoid) with 20% glycerol (Panreac, Badalona, Spain) for further studies. For sugar pattern tests

and identification experiments, isolates were cultured at 30°C in MRS broth for 24 h or on MRS agar for two to three days at 30°C.

Phenotypic characterisation

Identification of the isolates was done by comparing the phenotypic and biochemical characteristics of the strains with the previously published data (Schillinger and Lücke, 1987; Shaw and Harding, 1989; Collins et al., 1993; Villani et al., 1997). Phase contrast microscopy was used for examining the cell morphology. Growth at 8 and 15ºC was tested according to Schillinger and Lücke (1987) in tubes containing MRS broth and growth on Rogosa agar was tested on Rogosa agar plates (Oxoid) having the pH adjusted to 5.5 with glacial acetic acid (Panreac). The plates were incubated at 30ºC for 3 days under 6% of CO_2.

Fermentation of carbohydrates was determined according to the method described by Schillinger and Lücke (1987) using the miniplate method described by Jayne-Williams (1975) with the exception of using bromocresol purple as an indicator instead of chlorophenol red (Panreac) (Santos et al., 1998). Carbohydrates tested were D(+) cellobiose (Sigma, St. Louis, Missouri, USA), D(+) galactose (Sigma), inulin (Sigma), maltose 1-hydrate (Panreac), D manitol (Difco, Detroit, Michigan, USA), D(+) melezitose (Sigma), melibiose (Sigma), D(-) ribose (Sigma), salicin (Sigma), D(+) trehalose (Sigma), D(+) xylose (Merck, Darmstadt, Germany), and glucose (Panreac) and sterile water were used as positive and negative controls.

Gas production from glucose, dextran production from saccharose and hydrolysis of arginine were tested using the methods described by Schillinger and Lücke (1987) with the exception of adding glucose to the final concentration of 0.3 g/l to test NH_3 production from arginine. Production of acetoin was detected by the Voges-Proskauer test (Reuter, 1970). The configuration of lactic acid isomers was determined enzymatically (Roche Molecular Biochemicals, Mannheim, Germany) using supernatant from growth cultures incubated for 24h.

Ribotyping

*Hin*dIII restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA) was used for ribotyping. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (1989) as modified by Björkroth and Korkeala (1996a) by the combined lysozyme and mutanolysin (Sigma, St. Louis, Missouri, USA) treatment. Restriction endonuclease treatment of 3 µg of DNA was done as specified by the manufacturer (New England Biolabs) and REA as described before (Björkroth and Korkeala, 1996a). Before southern blotting, REA patterns were inspected visually in order to obtain preliminary information of the clonal variation. Genomic blots were made using a vacuum device (Vacugene, Pharmacia, Uppsala, Sweeden) and rDNA probe for ribotyping was labelled by reverse transcription (AMV-RT, Promega, Madison, Wisconsin, USA) and Dig DNA Labelling Kit (Roche) as previously described by Blumberg et al. (1991). Membranes were hybridised at 68ºC as described by Björkroth and Korkeala (1996a).

Pattern analysis: The *Hin*dIII ribopatterns were compared with the corresponding patterns in the previously established LAB database of the Department of Food and Enviromental Hygiene, University

of Helsinki, Finland. These comprise patterns of all relevant spoilage LAB in the genera of *Carnobacteria*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996b, 1997; Lyhs et al., 2000; Björkroth et al., 1998, 2000, 2002). For numerical analysis, ribopatterns were scanned using a Hewlett Packard (Boise, Idaho, USA) ScanJet 4c/T scanner and analysed using the GelCompar II software package (Applied Maths, Kortrijk, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation and UPGMA (unweighed pair group method using arithmetic averages) clustering was used for the construction of the dendrogram.

Results

According to the scheme by Schillinger and Lücke (1987), Shaw and Harding (1989) and Collins et al. (1993), 165 strains from the total of lactic isolates were phenotypically identified and 11 isolates with an uncertain identity were classified as *Lactobacillus spp.* The strains identified were divided in seven groups (A to G, Table 1). Heterofermentative bacteria (93.2%) were found to be the predominating LAB in “morcilla de Burgos”. Only 12 strains of the total isolates were homofermentative and they were included in groups F and G. All bacteria grew at 8 and 15ºC and only 6 strains (3.4%) produced acetoin. Most of the isolates grew on Rogosa agar except 9 strains of group A and 4 isolates of group G.

Heterofermentive rods, which did not hydrolyse arginine and fermented maltose but not galactose, were the major group isolated (42%) and these LAB were assigned to *Weissella viridescens* species (group A). Despite these bacteria produced both lactic acid isomers, concentrations of D(-) lactate were

almost twice the concentration of L(+) lactate isomer and only 5 strains were positive in the formation of dextran.

The 42 isolates (23.9%) of group B (second largest group) were assigned to the genus *Leuconostoc* since these isolates presented oval cocci growing in pairs, produced gas from glucose, did not hydrolyse arginine and formed D-lactate. Most of the strains from this group produced dextran from sucrose (71%) and none of them fermented inulin, mannitol and melezitose. This group was subdivided in three subgroups according to the diagnostics characteristics given by Shaw and Harding (1989), Collins et al. (1993) and Villani et al. (1997). Subgroup B1 included 20 strains (11.4% of the total LAB isolated) and they were described as *Lc. mesenteroides* due to the formation of dextran and fermentation of galactose, maltose, melibiose and trehalose. Isolates of subgroup B2 were identified as *Lc. carnosum* because of their inability to ferment galactose and xylose and the fermentation of trehalose. Almost half of the strains from this subgroup were dextran positive and more than 50 % of strains were not able to grow on Rogosa agar. Finally, 5 strains from leuconostoc group were not assigned to any species (subgroup B3) because the sugars tested and the fermentation patterns did not lead to a clear identification.

Strains in group C (11.4%) were identified as *Weissella confusa*. This group comprised 20 heterofermentative rod shaped isolates, arginine positive and highly dextran producers. These isolates formed DL lactate, however the amount of L(+) enantiomer was much higher than the other isomer for 14 strains from the total of this group.

The strains included in Group D were considered *Lactobacillus fructosus* due to the hydrolysis of arginine and the absence of fermentation for galactose and maltose. All isolates of this group presented a characteristic irregular rod shape, which was also found in 4 strains of group A and 2 strains of group E. The strains belonging to Group E were classified as *Lactobacillus sanfrancisco* in the basis of their gas production, the inability to ferment arginine and ribose and the fermentation of galactose. Production of D(-) lactate was higher than L(+) enantiomer production for groups D and E as it happened with strains of group B.

Group F comprised seven homofermentative strains with variable cell morphology. One of the bacteria presented coccobacilli shape, and this strain was considered belonging to genus *Pediococcus*. Five of the isolates were assigned to *Lactobacillus sakei* species and one to *Lactobacillus curvatus* species according to their characteristics.

Group G included five isolates characterised by being homofermentative rods, producing exclusively L(+) lactic acid form glucose and four of the five strains did not presented growth on Rogosa agar. They fermented mannitol and also cellobiose, maltose, ribose, salicin and trehalose. According to Schillinger and Lücke scheme (1987) this group corresponded to *Carnobacterium piscicola* (former *Lactobacillus carnis*).

Figure 1 shows the dendrogram and banding patterns of the isolates and the reference strains based on *Hin*dIII ribotypes. According to the results, 15 clusters were defined at a similarity level of 70%. The

ribotype of one isolate and the ribotypes of two reference strains (*Leuconostoc pseudomesenteroides* DSM 20193^T, LMG 11483) formed the cluster I at a similarity level of 78%. Cluster II was formed by 17 isolates (9.7%), which presented two different ribotypes. Fourteen strains had the same pattern than the type strain *Leuconostoc carnosum* NCFB 2776^T and the other isolates merged at the similarity level of 88% with the type strain mentioned. Cluster III included one isolate with the same ribopattern than *Leuconostoc citreum* (LMG 9824^T). Two strains with two different patterns and the reference strains *Leuconostoc lactis* (CCUG 30064^T, LMG 7940) formed the cluster IV. Cluster V contained a ribotype possessed by 19 strains (10.8%) and the reference strains *Lc. mesenteroides* subsp. *dextranicum* (LMG 17954, LMG 11318, DSM 20484^T) and *Lc. mesenteroides* subsp. *mesenteroides* (LMG 7939, DSM 20343^T). Cluster VI was the biggest one with 75 isolates (42.6%) and the type strain *Weissella viridescens* ATCC 12706^T.

Cluster VII had three different patterns of 17 isolates (9.7%), clustering at a similarity level of 88% but not reference strain pattern was found in this cluster. Cluster VIII was formed by one isolate and *Leuconostoc gasicomitatum* type strain (LMG 18811^T) merging at a similarity level of 84%. Cluster IX contained the ribotype of two isolates together with the type strains belonging to *Lactococcus lactis* species. One of the isolates and the type strain *L. Lactis* subsp. *lactis* (LMG 6890^T) merged at a similarity of 78% while the other one and the type strain *L. Lactis* subsp. *cremoris* (LMG 6897^T) merged at a similarity of 80%. Cluster X comprised the pattern of only one strain and the type strain *Lactococcus garvieae* (LMG 8893^T). Cluster XI was associated with 10 strains possessing five different ribotypes and the reference strains *Weissella cibaria* (LMG 17706, LMG 17704, LMG 17708, LMG 17699^T). Cluster XII contained the different types gained from 16 isolates, together with the type strains of *Weissella confusa* (LMG 9497^T, LMG 14040). Cluster XIII consisted of three different

patterns from 5 isolates and the pattern of the type strains *Lactobacillus sakei* subsp. *sakei* (ATCC 15521^T) and *Lactobacillus sakei* subsp. *carnosum*. Cluster XIV grouped 4 strains and type strain *Pediococcus pentosaceus* (LMG 11488^T). Finally Cluster XV was formed by the pattern of one strain and the type strain *Lactobacillus curvatus* subsp. *curvatus* (ATCC 25601^T) merging at a similarity level of 84%.

The results of the strains identified from both methods are shown in table 2. According to the results most of the strains included in the species *W. viridescens*, *Lc. mesenteroides* y *Lc. carnosum*, the strains *Lb. sakei* and *Lb. curvatus*, and half of the strains *W. confusa* were correctly classified by both methods (around 70% of the total isolates). However ribotyping identification do not consider the presence of *Lb. fructosus*, *Lb. sanfrancisco* and *Carnobacterium piscicola* and establish the presence of other species like *W. cibaria*, species from *Lactococcus* genus and also a group of bacteria which has not been identified.

Table 3 shows the final distribution of the strains according to their origin. *Weissella viridescens* was the major group present in isolates from "morcilla" from different producers, unpacked, modified atmosphere packaged and pasteurised "morcilla" with percentages above 40%. On the contrary, *W. confusa*, *W. viridescens* and *Lc. mesenteroides* were the dominant LAB in spoiled vacuum packaged "morcilla" with similar percentages (29, 26 and 26%). *Lc. mesenteroides* was also important in unpacked and pasteurised "morcilla" with percentages of 16 and 17%, respectively. *Lc. carnosum* was the second important group in modified atmosphere packaged "morcilla" although it was present also in paper wrapped and vacuum packaged product. Apart from *W. viridescens* (isolated in product from 8

271 factories) strains from *W. cibaria* and cluster VII were isolated from “morcillas” from different
272 producers, specifically from four and five factories, respectively.

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275 **Discussion**

276 The combination of phenotypic and genotypic methods led to a better identification and
277 characterization of the strains isolated from “morcilla de Burgos”. Many authors have reported the
278 difficulty of identification of leuconostocs by phenotypic means due to the great heterogeneity in
279 biochemical and physiological characteristics (Milliere et al., 1989; Shaw and Harding, 1989; von Holy
280 et al., 1991; Mäkelä et al., 1992; Dykes et al., 1994a; Björkroth et al., 1998; Samelis et al., 2000a).
281 Even differentiation of the leuconostocs from the atypical heterofermentative arginine negative
282 lactobacilli (like *W. viridescens* and *Lb. fructosus*) using phenotypic criteria is often very difficult due
283 to the fact that morphology can lead to mistakes and these bacteria produce predominantly D(-) lactic
284 acid isomer (Collins et al., 1993).

285

286 In this case most leuconostocs were correctly classified by phenotypic means. However, ribotyping
287 revealed also the presence of species like *Lc. pseudomesenteroides*, *Lc. lactis*, *Lc. carnosum*, *Lc.*
288 *citreum* and *Lc. gasicomitatum*. The latest species has been recently described by Björkroth et al.
289 (2000) in spoiled raw tomato-marinated broiler meat strips packaged under modified-atmosphere
290 conditions. Although isolates from group B1 (*Lc. mesenteroides*) were not assigned to any subspecies,
291 these bacteria resembled more *Lc. mesenteroides* subsp. *mesenteroides* and *Lc. mesenteroides* subsp.

292 *dextranicum* than *Lc. mesenteroides* subsp. *cremoris* since our isolates were dextran positive and
293 fermented more sugars than galactose (Shaw and Harding, 1989; Milliere et al., 1989; Collins et al.,
294 1993; Villani et al., 1997). These facts were confirmed by ribotyping analyses (see Figure 1).

295

296 The group phenotypically classified as *W. confusa* really comprised two species (*W. confusa* and *W.*
297 *cibaria*) according to genotyping (see Figure 1). These *W. cibaria* isolates unlike strains of *W. confusa*
298 presented a weak fermentation of xylose and did not ferment ribose. This species has been recently
299 described by Bjorkroth et al. (2002).

300

301 Species belonging *Lactococcus* clusters according to ribotyping results had been phenotypically
302 misidentified as *Carnobacterium piscicola* due to the fact that they presented rod shape at the
303 microscopy. Different works have reported that electronic microscopy offers better results in the
304 determination of the shape of this genus than phase contrast microscopy (Mauguin and Novel, 1994;
305 Barakat et al., 2000). Although this genus is traditionally associated to dairy and vegetable products,
306 species from *L. lactis* and *L. garvieae* have been isolated from fermented sausages (Rodríguez et al.,
307 1995), pork meat (Garver and Muriana, 1993) and poultry meat (Barakat et al., 2000).

308

309 *Lb. fructosus* and *Lb. sanfrancisco* species (groups D and E) are quite similar to *W. viridescens*
310 according to Schillinger and Lücke scheme (1987) and are rarely found in meat and meat products
311 which is confirmed by ribotyping (Table 3). Cluster VII comprised the isolates with irregular shape
312 belonging to *Lb. fructosus*, *Lb. sanfrancisco* and *W. viridescens* species by phenotypic means. This

group of LAB could be a new variant of *W. viridescens* species or a different species but more information like DNA homology studies and whole-cell protein analysis is necessary in order to confirm the identity of these strains.

The majority of the LAB associated with morcilla produced gas from glucose. In fact, homofermentative LAB, especially *Lb. sakei/curvatus* were hardly present although these species have usually been referred as the main spoilage microorganisms in vacuum and modified atmosphere packed meat and meat products (Hitchener et al., 1982; Morishita and Shiromizu 1986; Borch et al., 1996; Samelis et al., 2000a, 200b).

The high presence of heterofermentative bacteria can be considered to be responsible for the abundant blowing of the packs observed in the case of “morcilla” packed in vacuum or modified atmosphere. The proportion of heterofermentative LAB is clearly higher in “morcilla” compared to the LAB found by other authors in meat and meat products (Shaw and Harding, 1984; Morishita and Shiromizu, 1986; Schillinger and Lücke, 1987; Korkeala and Mäkelä, 1989; von Holy et al., 1992; Dykes et al., 1994a; Franz and von Holy, 1996; Samelis et al., 2000a, 2000b). The presence of heterofermentative LAB (lactobacilli and leuconostocs) in the previous studies was lower than 50%. Hitchener et al. (1982) found a high level of heterofermentative bacteria (75%) although in this work most of the isolates were L-lactate producers and they called them as atypical Betabacteria, which were later identified as *Carnobacterium* species (Shaw and Harding, 1985). Björkroth et al. (2000) found *Leuconostoc gasicomitatum* sp. nov. dominating (57% of the total LAB microflora) in a tomato-marinated, raw broiler meat strip product packaged under modified atmosphere. This product had also been showing

gaseous spoilage and extensive bulging of the packages. Heterofermentative rods and leuconostocs were also detected as significant part of bacterial microflora in unspoiled vacuum packaged smoked Vienna sausages (von Holy et al., 1991).

W. viridescens was the major LAB found in fresh and modified atmosphere packaged “morcilla” as well as pasteurized “morcilla”. This species has especially been associated with the greening of meat products due to the production of hydrogen peroxide (Niven and Evans, 1957; Hammes et al., 1991) but lower numbers of *W. viridescens* have been found by other workers both in fresh meat and meat products (Morishita and Shiromizu, 1986; Schillinger and Lücke, 1987) and cooked products (Samelis et al., 2000a, 2000b). This species has occasionally been observed to form the main spoilage populations in Swedish ring sausages (Borch et al., 1988).

Leuconostocs and specially *Lc. mesenteroides* and *Lc. carnosum* species are commonly found in spoiled vacuum meat and meat products (Shaw and Harding, 1989; Korkeala and Mäkelä, 1989; von Holy et al., 1991, 1992; Dykes et al., 1994b; Franz and von Holy, 1996; Björkroth et al., 1998; Samelis et al., 2000a; 2000b). When the product was vacuum packaged the proportion of leuconostocs increased in the spoiled product. This phenomenon was also observed by Samellis et al. (2000a) in sliced vacuum packed, unsmoked boiled turkey breast fillets, where *Lc. mesenteroides* subsp. *mesenteroides* were the predominant species at the last steps of storage, while *W. viridescens* was the main species of the initial microflora. Samelis et al. (2000b) also reported the prevalence of the *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Lc. carnosum* in vacuum and air packed cooked

356 ham, and in turkey fillets while *W. viridescens* was found at low numbers in vacuum-packed smoked
357 pork loin, bacon, “pariza” and “mortadella” as well as in vacuum and air-packed frankfurters.

358

359 It is quite interesting the presence of *W. confusa* and *W. cibaria* in the spoilage microbiota of ”morcilla
360 de Burgos”. These two species has been diferentiated only recently by Björkroth et al. (2002), who
361 described the presence of these species in Malaysian foods and in clinical samples from humans and
362 animals. Morishita and Shiromizu (1986) also reported the presence of *W. confusa* in meat and meat
363 products but no more references have been found about the presence of this species.

364

365 The fact that *W. viridescens* was the main group of bacteria that survived to the pasteurisation treatment
366 confirms the findings made by other authors in cooked meat products which consider this species as a
367 heat resistant microorganism (Niven et al., 1954; Milbourne, 1983; Borch et al., 1988).

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369 According to the origin of the isolates, the microbiota in “morcilla” from different producers was a
370 little more diverse but the main species found confirmed that microbiota associated to “morcilla de
371 Burgos” is product characteristic and is not related to the origin of the factory. The different lactic
372 microbiota described in “morcilla” can be attributed to the different raw materials employed in its
373 manufacture as onion, rice and blood and the absence of curing salts, which might favour the
374 development of heterofermenters as contrasted with the species habitually found in emulsion sausages.
375 In this way, raw material could be thought the source of spoilage LAB that contaminates the product

during handling after cooking step. However, more information about contamination sources is necessary to confirm this hypothesis.

Conclusion

Phenotypic characterization based on sugar fermentation pattern and conventional phenotypic properties may not always provide sufficient basis for the reliable identification of LAB, although it is a useful tool for presumptive classification. In this way, ribotyping was really useful for the identification of LAB from “Morcilla de Burgos”, although few isolates remained unclassified and could be a new species (cluster VII). It can be concluded that *W. viridescens, Lc mesenteroides, Lc. carnosum* and *W. confusa* are the main members of LAB in “morcilla de Burgos”. During cold storage development of *Leuconostoc* species is favoured in vacuum packaged samples while *W. viridescens* is predominant when the product is pasteurised after packing.

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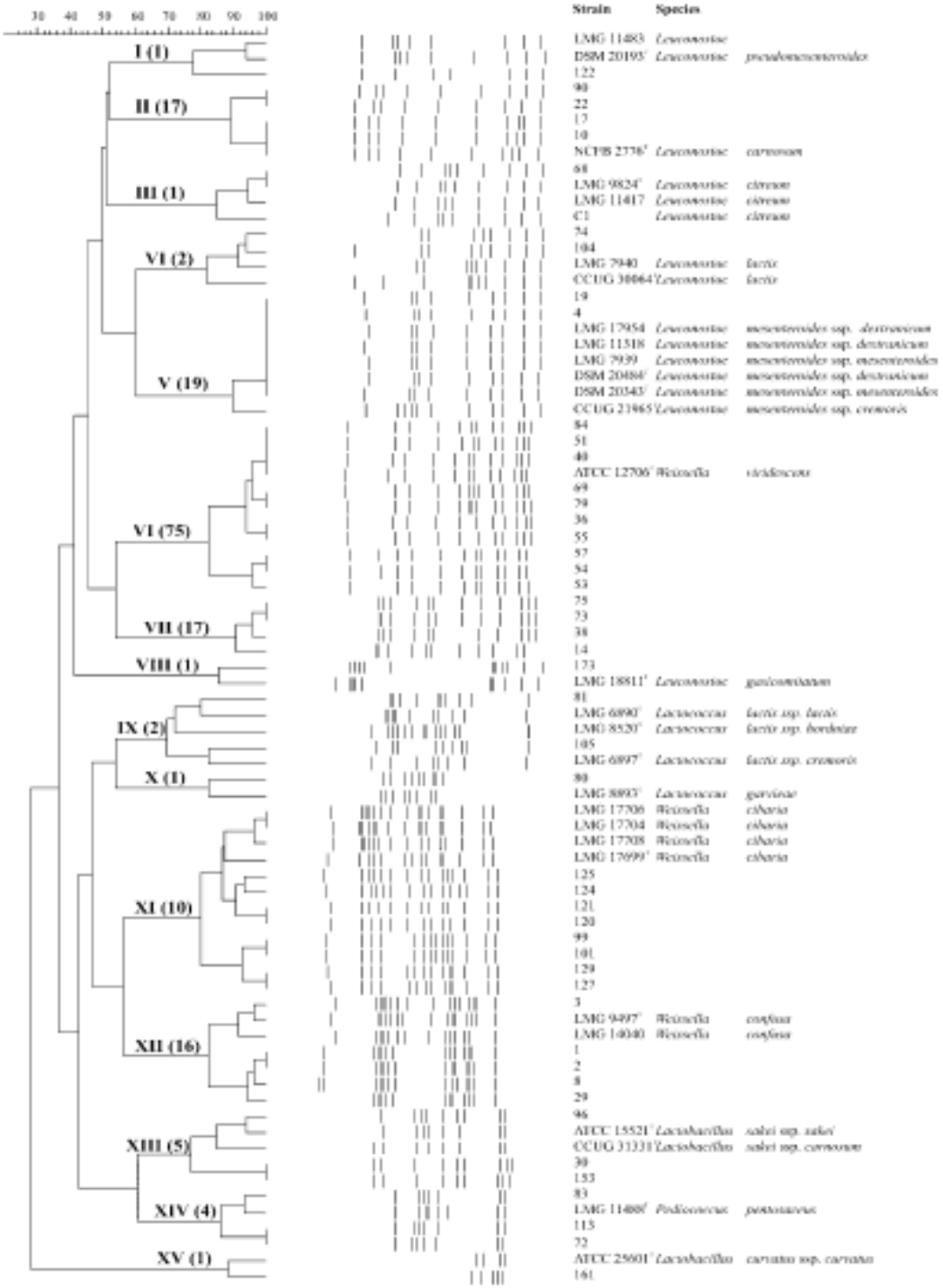
Figure Captions

Figure 1. Phylogenetic tree of lactic acid bacteria isolated from ”morcilla de Burgos” by ribotyping. (I-XV) cluster number. Numbers in brackets are the number of strains analysed per cluster.



522 Table 1. Characteristics of the LAB groups obtained.

	LAB groups						
	A	**B**	**C**	**D**	**E**	**F**	**G**
Nº of strains	74	42	20	10	7	7	5
Percentage	42.0	23.9	11.4	5.7	4.0	4.0	2.8
Cell morphology	Regular rods	Cocco-bacilli	Short rods	Irregular rods	Regular rods	Rods/ cocco-bacilli	Short rods
Gas production	+[a]	+	+	+	+	–	–
NH_3 from arginine	–	–	+	–	–	71[b]	+
Dextran formation	7	71	+	–	–	–	–
Voges Proskauer test	–	–	–	–	–	43	60
Lactic acid configuration	DL	D	DL	DL	DL	L	L
Growth:							
at 8ºC	+	+	+	+	+	+	+
at 15ºC	+	+	+	+	+	+	+
on Rogosa agar	(+)	79	+	+	+	+	20
Acid produced from:							
Cellobiose	5	50	+	10	71	86	+
Galactose	–	(50)	85	–	+	+	60
Inulin	–	–	–	–	–	–	40
Maltose	+	60	+	–	+	43	+
Mannitol	–	–	–	–	–	–	+
Melezitose	–	–	–	–	–	–	40
Melibiose	4	55	–	–	+	71	–
Ribose	85	(90)	(50)	–	71	+	+
Salicin	–	50	+	–	71	+	+
Trehalose	88	98	–	+	+	86	+
Xylose	4	50	(+)	–	–	–	40

523 [a] Symbols: +: all strains positive; –: all strains negative; [b]: % of positive strains; ( ): some strains weak
524 reaction.

525 Table 2. Phenotypic and genotypic identification of LAB isolates from "morcilla de
526 Burgos".

No. of isolates	Phenotypic identification	No. of isolates	Ribotyping	Code[a]
74	*W. viridescens* (A)[b]	68	*W. viridescens*	C
		4	Cluster VII	I
		1	*Lc. gasicomitatum*	I
		1	NT[c]	
20	*Lc. mesenteroides* (B1)	19	*Lc. mesenteroides*	C
		1	*Lc. pseudomesenteroides*	U
17	*Lc. carnosum* (B2)	15	*Lc. carnosum*	C
		2	NT	
5	*Leuconostoc spp.* (B3)	2	*Lc. lactis*	U
		1	*Lc. carnosum*	U
		1	*Lc. citreum*	U
		1	NT	
20	*W. confusa* (C)	11	*W. confusa*	C
		9	*W. cibaria*	U
10	*Lb. fructosus* (D)	10	Cluster VII	I
7	*Lb. sanfrancisco* (E)	5	*W. viridescens*	I
		2	Cluster VII	I
1	*Pediococcus spp* (F)	1	*Pediococcus pentosaceus*	U
5	*Lb. sakei* (F)	5	*Lb. sakei*	C
1	*Lb. curvatus* (F)	1	*Lb. curvatus*	C
5	*C .piscicola* (G)	2	*Lactococcus lactis*	I
		1	*Lactococcus garvieae*	I
		2	*Pediococcus pentosaceus*	I
11	*Lactobacillus sp.*	5	*W.confusa*	I
		2	*W. viridescens*	I
		1	*W. cibaria*	I
		1	*Pediococcus pentosaceus*	I
		1	*Lc. carnosum*	I
		1	Cluster VII	I

527 [a]: **C**: genus and species correctly identified by phenotypic methods; **U**: same genus,
528 but different species identified by phenotypic methods and **I**: different genus and
529 species identified by phenotypic methods.
530 [b]: ( ): Phenotypic group.
531 [C]: NT: not tested by ribotyping.

532 Table 3. Distribution of the strains according to the origin of the isolates. Percentages are in brackets.

	"Morcilla" from different producers	Paper wrapped "morcilla"	Vacuum packed "morcilla"	MAP "morcilla"	Pasteurised "morcilla"	Total
Nº of strains	66	37	31	24	18	176
W. viridescens	27 (41)	17 (46)	8 (26)	13 (54)	11 (61)	76 (43)
Lc. mesenteroides	2 (3)	6 (16)	8 (26)	–	3 (17)	19 (11)
Lc. carnosum	2 (3)	5 (14)	4 (13)	8 (33)	–	19 (11)
Other leuconostocs	4 (6)	–	–	1 (4)	–	5 (3)
Cluster VII	9 (14)	5 (14)	2 (6)	–	1 (6)	17 (10)
W. confusa	1 (2)	4 (11)	9 (29)	–	2 (11)	16 (9)
W. cibaria	10 (15)	–	–	–	–	10 (6)
Lb. sakei/Lb. curvatus	3 (5)	–	–	2 (8)	1 (6)	6 (3)
Pediococcus pentosaceus	4 (6)	–	–	–	–	4 (2)
Lactococcus spp.	3 (5)	–	–	–	–	3 (2)
NI[a]	1 (2)	–	–	–	–	1 (1)

533 [a]: NI: not identified
534
535